

Arch Microbiol (2001) 176:37–43  
DOI 10.1007/s002030100290

## ORIGINAL PAPER

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# Structures of coenzyme $F_{420}$ in *Mycobacterium* species

Received: 8 January 2001 / Revised: 5 April 2001 / Accepted: 9 April 2001 / Published online: 22 May 2001

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**Abstract** The structure of coenzyme  $F_{420}$  in *Mycobacterium smegmatis* was examined using proton NMR, amino acid analysis, and HPLC. The two major  $F_{420}$  structures were shown to be composed of a chromophore identical to that of  $F_{420}$  from *Methanobacterium thermoautotrophicum*, with a side chain of a ribityl residue, a lactyl residue and five or six glutamate groups ( $F_{420-5}$  and  $F_{420-6}$ ). Peptidase treatment studies suggested that L-glutamate groups are linked by  $\gamma$ -glutamyl bonds in the side chain. HPLC analysis indicated that *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, and *Mycobacterium fortuitum* have  $F_{420-5}$  and  $F_{420-6}$  as the predominant structures, whereas *Mycobacterium avium* contains  $F_{420-5}$ ,  $F_{420-6}$  and  $F_{420-7}$  in significant amounts. 7,8-Didemethyl 8-hydroxy 5-deazariboflavin (FO), an intermediate in  $F_{420}$  biosynthesis, accounted for about 1–7% of the total deazaflavin in cells. Peptidase treatment of  $F_{420}$  created  $F_{420}$  derivatives that may be useful for the assay of enzymes involved in  $F_{420}$  biosynthesis.

**Keywords** Coenzyme  $F_{420}$  · FO · *Mycobacterium* · Polyglutamate · 5-deazaflavin · Folate

## Introduction

$F_{420}$  is a two-electron transfer coenzyme that is present in some members of three distantly related groups of prokaryotes: the archaea, the aerobic actinomycetes, and the cyanobacteria. Very few other genera contain  $F_{420}$ . In archaea,  $F_{420}$  is essential for hydrogenase, formate dehydrogenase, methylene-tetrahydromethanopterin dehydrogenase, alcohol dehydrogenase, methylene-tetrahydromethanopterin reductase, and quinone oxidoreductase reactions

(Jones and Stadtman 1980; Jacobson et al. 1982; Hartzell et al. 1985; Widdel and Wolfe 1989; DiMarco et al. 1990; Ma and Thauer 1990; Daniels 1994; Kunow et al. 1994).  $F_{420}$  and/or 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO, a biosynthetic precursor of  $F_{420}$ ) is used by *Streptomyces* species in tetracycline and lincomycin biosynthesis (Rhodes et al. 1981; McCormick and Morton 1982; Coats et al. 1989) and may be used in mitomycin C biosynthesis (Mao et al. 1999). In *Mycobacterium* and *Nocardia*,  $F_{420}$  is used by  $F_{420}$ -dependent glucose-6-phosphate dehydrogenase (Purwantini and Daniels 1996; Purwantini et al. 1997). The cyanobacteria *Synechococcus* sp. (previously known as *Anacystis nidulans*) and *Synechocystis* sp., and the green alga *Scenedesmus acutus* use  $F_{420}$  in their photolysis (Eker et al. 1988, 1990; Ng et al. 2000).

The structure of coenzyme  $F_{420}$  from *Methanobacterium thermoautotrophicum* ( $F_{420-2}$ ), shown in Fig. 1A, contains a FO chromophore (Eirich et al. 1978, 1979). The side chain attached at the N-10 position contains ribityl, phosphate, and lactyl groups in sequence, terminating with two L-glutamate groups connected with a  $\gamma$ -glutamyl linkage. FO, a biosynthetic intermediate that lacks the phosphate-to-glutamate portion of the side chain, is found in cells and media of methanogens and actinomycetes (McCormick and Morton 1982; Kern et al. 1983; Kuo et al. 1989). *Methanosarcina barkeri* makes modified  $F_{420}$  structures thought to contain both four and five  $\gamma$ -linked glutamate groups (Gorris and van der Drift 1986; Peck 1989); however, no primary data have been published to establish the number of glutamate residues, and it has not been determined if they are linked by  $\alpha$ - or  $\gamma$ -bonds. Precise structural work on *Streptomyces* 5-deazaflavins has been limited to FO (McCormick and Morton 1982; Kuo et al. 1989), but thin-layer electrophoresis of *Streptomyces griseus*  $F_{420}$  has suggested that more than two glutamate residues are present (Eker 1980).  $F_{420}$  from *Sulfolobus*, *Thermoplasma*, and halobacteria is reported to contain two or more glutamate groups, based on TLC (Lin and White 1986).  $F_{420}$  from *Mycobacterium avium* is reported to have one glutamate group, based on amino acid analysis (Naraoka et al. 1984).

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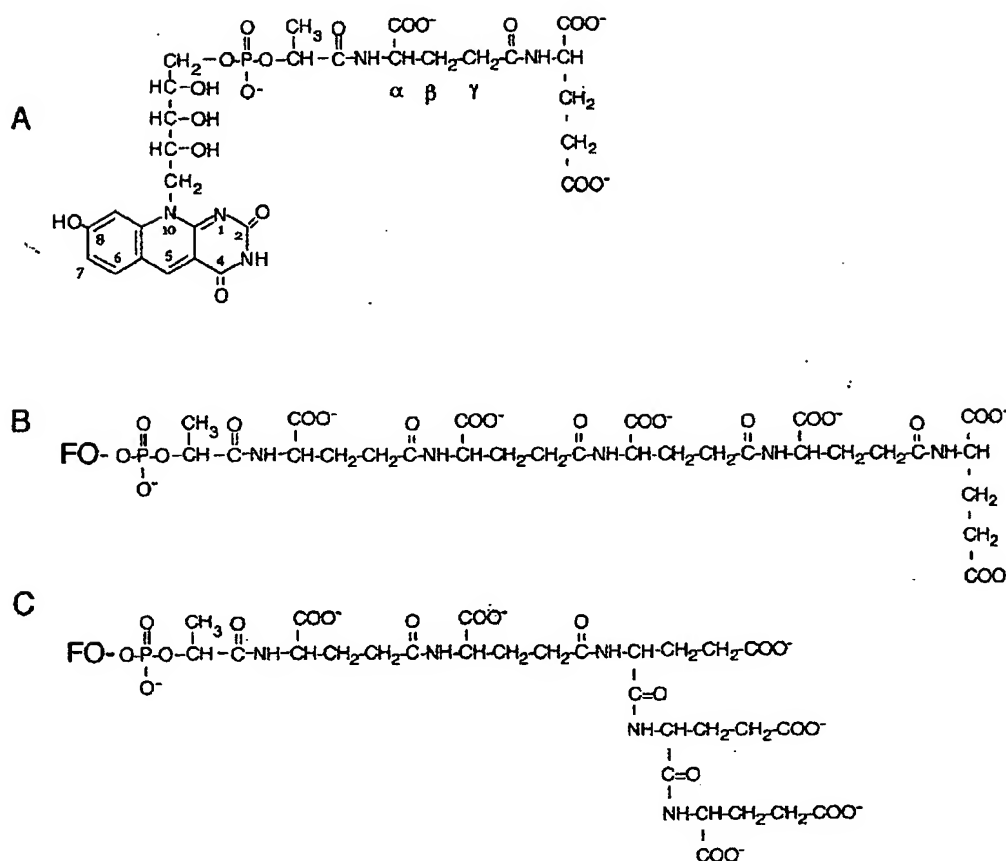


Fig. 1A-C Structure of  $F_{420}-2$  from *Methanobacterium thermoautotrophicum*, and possible structures of the side chain in  $F_{420}-5$  from *Mycobacterium* species. A *Methanobacterium thermoautotrophicum*  $F_{420}-2$ , with the second glutamate group linked by a  $\gamma$ -glutamyl bond. B Structure of pentaglutamate sidechain bound to the lactyl of  $F_{420}$  with the second to fifth glutamate residues linked by  $\gamma$ -glutamyl bonds, in analogy with eukaryotic folate; C Structure of pentaglutamate side chain with the second and third glutamate groups linked by  $\gamma$ -glutamyl bonds, but the fourth and fifth glutamate residues linked by  $\alpha$ -glutamyl bonds, in analogy with *Escherichia coli* folate

The side chain of  $F_{420}$  is structurally analogous to the side chain of folic acid since glutamate groups are attached to the carboxyl of the lactyl moiety of the  $F_{420}$  instead of the carboxyl of the *p*-aminobenzoic moiety of folate. Both eukaryotes and prokaryotes contain folic acid with two to seven glutamate groups (Blakley and Benkovic 1984). All glutamate groups in eukaryotic folic acid are joined by a  $\gamma$ -glutamyl bond, whereas in *Escherichia coli* the second and third glutamate groups are linked by  $\gamma$ -glutamyl linkages, and the remaining glutamate groups are linked by  $\alpha$ -glutamyl bonds (Ferone et al. 1986; Johnson et al. 1988). Sarcinopterin, an analogue of folic acid in *Methanosarcina barkeri*, contains a glutamate attached via an amide bond to the  $\alpha$ -carboxylic acid

group of the hydroxyglutaric moiety of methanopterin (van Beelen et al. 1984), which establishes precedence for an  $\alpha$ -glutamyl linkage in an organism that makes  $F_{420}$ .

Our initial HPLC analysis of *Mycobacterium smegmatis* suggested that several polyglutamate  $F_{420}$  species were present. The glutamate groups could have been linked by either  $\alpha$ - or  $\gamma$ -glutamyl linkages. As depicted in Fig. 1B, a pentaglutamate  $F_{420}$  could be analogous to eukaryotic folate (with all glutamate groups linked with the  $\gamma$ -carboxyl of the prior glutamate). Alternatively, as in Fig. 1C,  $F_{420}$  could be analogous to *E. coli* folate (with the terminal two glutamate groups linked to the  $\alpha$ -carboxyl of the prior glutamate), or any mixture of glutamyl bonds may have been present. We report here that the predominant  $F_{420}$  in all *Mycobacterium* species examined contain four to seven glutamate molecules, unlike the monoglutamate structure proposed earlier for *M. avium*  $F_{420}$  (Naraoka et al. 1984), and we also provide evidence that all the glutamate molecules are the L-isomer, and are all linked by  $\gamma$ -glutamyl bonds.

## Materials and methods

### Bacterial strains, media, and growth conditions

*Mycobacterium smegmatis* mc<sup>2</sup>155 was a gift of Dr. W. Jacobs Jr. (Howard Hughes Medical Institute, Albert Einstein College of Medicine, New York). *M. avium* ATCC 25291, *Mycobacterium bovis* BCG (Montreal) ATCC 35746, and *Mycobacterium fortuitum* ATCC 6841 were obtained from the ATCC (Manassas, Va.). The above mycobacteria were typically grown in Middlebrook 7H9 medium (Difco) supplemented with 0.2% glycerol and 0.05% Tween 80, except that Bacto Middlebrook ADC Enrichment was added for growth of *M. bovis* and *M. avium*. To test the role of glutamate in the medium on F<sub>420</sub> structure, *M. smegmatis* was grown in two other media. One contained a high level of glutamate (g per liter): K<sub>2</sub>HPO<sub>4</sub> (0.5), MgSO<sub>4</sub> (1), sodium citrate (2), ferric ammonium citrate (0.05), sodium glutamate (40), Tween 80 (0.5), and glycerol (60) (Naraoka et al. 1984). Another medium with no glutamate contained (g per liter): NaH<sub>2</sub>PO<sub>4</sub> (3.44), KH<sub>2</sub>PO<sub>4</sub> (4.0), MgSO<sub>4</sub>·7H<sub>2</sub>O (2.5), sodium citrate 2H<sub>2</sub>O (2.5), ferrous ammonium sulfate (0.01), L-asparagine (5.0), glycerol (25) (Bowles and Segal 1965). *M. tuberculosis* strains H<sub>37</sub>Rv and CSU93 were grown at Colorado State University by Dr. John Belisle using glycine-alanine-salts medium in flasks with gentle agitation for about 14 days. *Methanobacterium thermoautotrophicum* strain Marburg was grown in a 100-l fermentor on H<sub>2</sub>-CO<sub>2</sub> (80:20, v/v), and *Methanosarcina barkeri* was grown in a carboy on methanol as described previously (Daniels 1995).

A richer medium was used to grow *M. smegmatis* for large-scale preparation of F<sub>420</sub> for NMR analysis. This medium contained (g per liter): glucose (15), blackstrap molasses (20), soluble starch (40), cottonseed hydrolysate (25), CaCO<sub>3</sub> (8), and K<sub>2</sub>SO<sub>4</sub> (2) (Kuo et al. 1989). After 8–9 days of growth (750 ml in 2.8-l Fernbach flasks), the cells were harvested, washed in distilled water and the pellet was frozen until use.

### HPLC methods

Different forms of F<sub>420</sub> were separated by HPLC using a Beckman System Gold 126 HPLC with a Shimadzu RF-10Axi fluorescence detector (excitation at 400 nm, emission at 470 nm). Analytical HPLC was carried out using a method similar to that of Gorris and van der Drift (1988), with modifications. A 3.9×300 mm α-Bond-pack C-18 (Supelco) column was eluted at 1 ml/min. Buffer A was 27.5 mM sodium acetate (pH 4.7) containing 2% acetonitrile; buffer B was 100% acetonitrile. The portion of buffer B in the elution buffer was varied as follows: 0–2 min, 0%; 2–6 min, 0–2%; 6–15 min, 2–10%; 15–22 min, 28%; 22–27 min, 28–0% (all linear gradients). F<sub>420</sub> prepared for NMR spectroscopy was further purified by two semi-preparative HPLC methods with a 10×250 mm Supelcosil LC-18 column eluted at 3 ml/min. The first method was identical to the analytical method except for flow rate and column. The second was isocratic methanol:water (20:80, v/v). The product of F<sub>420</sub> degradation by carboxypeptidase G was purified by HPLC using no sodium acetate for the acetonitrile elution.

### Purification of F<sub>420</sub> and FO

*Mycobacterium smegmatis* from 3 l of molasses medium culture was extracted by boiling in 150 ml of 50 mM sodium phosphate buffer (pH 7) for 30 min. After centrifugation and removal of the supernatant, the pellet was reextracted with 150 ml buffer and the supernatants were combined and filtered with a 0.45-μm filter. The extract was applied in three separate loadings of ~110 ml each to a QAE-Sephadex A-25 column (5×12 cm) equilibrated with 50 mM sodium phosphate (pH 7). FO and F<sub>420</sub> were eluted with a 0–1 M NaCl gradient (total volume, 1,400 ml) in the same buffer using a Biorad chromatography system and a fluorescence detector (Gilson Model 121, Middleton, Wis.). FO and F<sub>420</sub> gave two well-

resolved fluorescent peaks that were collected separately. These separate peaks were adjusted to pH 4.7 and loaded onto a 1.5×7 cm column packed with Sephasil C18 (Pharmacia Biotech) that had been equilibrated with 25 mM sodium acetate, pH 4.7. FO and F<sub>420</sub> were eluted with a 0–80% methanol gradient in the same buffer (total volume, 280 ml). Peaks from this column were adjusted to pH 7 and run on a second QAE column (1.5×15 cm). The amount of F<sub>420</sub> (4.05 mg) and FO (1.7 mg) from the 3 l of culture were estimated by A<sub>420</sub> (ε<sub>420</sub>=38.5 mM<sup>-1</sup> cm<sup>-1</sup>, pH 7.0). A portion of this F<sub>420</sub> was then chromatographed by semi-preparative HPLC using both the acetonitrile and methanol-water techniques described in the HPLC methods. F<sub>420</sub> from *Methanobacterium thermoautotrophicum* was purified with the same procedures.

### NMR and amino acid analysis

<sup>1</sup>H-NMR spectra were obtained on a Varian Inova 500 MHz spectrometer in the University of Iowa College of Medicine NMR facility. Up to 2,048 transients were averaged using a 6-s relaxation delay, a 6.9-μs (90°) pulse width and a 2.73-s acquisition time. The spectral width was 24 ppm, centered on the HDO peak at 4.757 ppm. The HDO signal was suppressed by continuous wave presaturation during the recycle delay; 32,768 complex points were collected, zero filled to 128 K and Fourier-transformed using a 0.5-Hz Lorentzian line broadening. Spectra were baseline-corrected using a spline function before peak integrals were measured. Samples were analyzed in Wilmad 535 tubes using Dory susceptibility-matched volume-reducing plugs (Wilmad Glass, Buena, N.J.).

F<sub>420</sub> and the product of carboxypeptidase G F<sub>420</sub> degradation were hydrolyzed at 110°C in 6 N HCl for 24 h, then analyzed by the UI Molecular Analysis Facility for amino acid content by HPLC with a cation exchange column and conventional automated ninhydrin analysis.

### Peptidase reactions

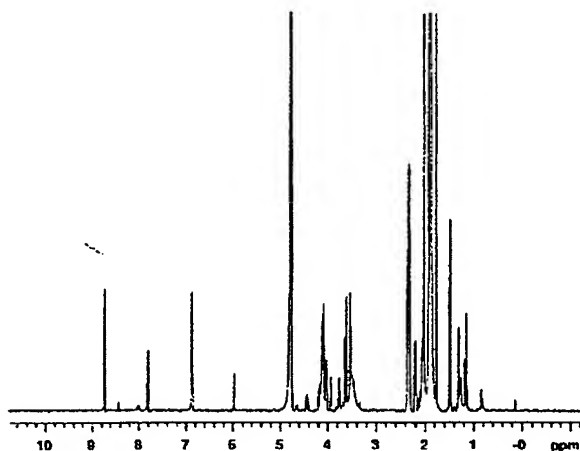
*Mycobacterium smegmatis* F<sub>420</sub> (6.5 μg) was incubated with peptidases to determine if the glutamate chains were degraded. The carboxypeptidase Y reaction was carried out by mixing F<sub>420</sub> with 100 μl of 25 mM sodium citrate (pH 5.5) and 4–6 units of carboxypeptidase Y (Sigma or ICN Biomedicals, both from bakers yeast). The carboxypeptidase G reaction was carried out by mixing F<sub>420</sub> with 100 μl of 25 mM Tris-HCl (pH 7.3) and 1.25–4 units of carboxypeptidase G (Sigma, from *Pseudomonas*). The γ-glutamyl-transpeptidase reaction was carried out by mixing F<sub>420</sub> with 50 μl of 25 mM ammonium acetate (pH 8.8) and 50 μl of γ-glutamyl-transpeptidase (4–8 units; equine or bovine kidney, Sigma) dissolved in 0.1 M Tris-10 mM MgCl<sub>2</sub> (pH 9.0). The reactions were started by the addition of F<sub>420</sub>, and incubated at 30°C. Samples were taken at various times between 0 and 50 h, boiled, centrifuged to remove precipitated protein, and frozen until analyzed by HPLC. Reactions with boiled enzymes were used as controls.

## Results and discussion

### NMR analysis of F<sub>420</sub>

Figure 2 shows the proton NMR spectrum of F<sub>420</sub> from *M. smegmatis*. This F<sub>420</sub> was the major peak purified with preparative HPLC, and showed two partially overlapping peaks when examined by analytical HPLC. Table 1 provides a quantitative comparison of these F<sub>420</sub> NMR data with data collected with F<sub>420</sub>-2 from *Methanobacterium thermoautotrophicum*. Overall, our NMR data on F<sub>420</sub> and FO are in agreement with the data of Eirich et al. (1978)

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Fig.2 Proton NMR spectrum of *Mycobacterium smegmatis* F<sub>420</sub>

observed at 2.34 ppm (from the glutamate  $\gamma$ -methylene group, indicated in Fig.1A), which corresponds to 5.03 glutamate molecules; a repeat of this NMR analysis resulted in an average of 5.3 glutamate molecules. NMR of this region with the methanogen F<sub>420</sub> revealed 4.17 protons, consistent with the two glutamate molecules expected. Analysis of the *M. smegmatis* F<sub>420</sub> region of 3.8–5.1 ppm, which should represent a mixture of ribityl group protons and glutamate methine protons, was complicated by possible errors in subtracting the water peak from this region, but was consistent with the presence of the seven non-hydroxyl ribityl group protons and four glutamate molecules. The strong signal at 1.85, and its associated sidebands, arise from the methyl group of acetate, which remained as a contaminant after its use in HPLC purification of F<sub>420</sub>.

#### Amino acid analysis of F<sub>420</sub>

and Kuo et al. (1989), and we have used their assignments as a guide for our work. The aromatic protons in positions C-5, C-6, C-7, and C-9 were approximately one each in F<sub>420</sub> from both sources, suggesting that the chromophore is identical in both organisms. This was in agreement with our proton NMR analysis of *M. smegmatis* FO, and with UV-visible spectra for F<sub>420</sub> from mycobacteria and methanogens (data not shown). The signal at 1.49 ppm yielded 2.78 protons for *M. smegmatis* F<sub>420</sub>, which matched well with the corresponding methanogen F<sub>420</sub> signal of 3.07 protons, indicating the presence of one lactyl methyl group. An accurate measure of the number of glutamate molecules present was obtained with the 10.06 protons

Amino acid analysis of F<sub>420</sub> indicated that 5.20 glutamate molecules were present per molecule of *M. smegmatis* F<sub>420</sub>. Small amounts of glycine (less than one molecule per F<sub>420</sub> molecule) were generally seen, and smaller amounts of lysine were occasionally seen. Both are likely due to trace contaminants from sample processing. These data, coupled with NMR analysis (above) and HPLC analysis (below), indicate that the two species in the sample analyzed are principally F<sub>420</sub>-5 and F<sub>420</sub>-6, averaging to "F<sub>420</sub>-5.3."

Table 1 Proton NMR data for F<sub>420</sub> purified from *Mycobacterium smegmatis* and *Methanobacterium thermoautotrophicum*. (s) Singlet, (d) doublet, (m) multiplet

F <sub>420</sub> source	Carbon atom	Chemical shift in ppm	Protons <sup>a</sup>	
			Observed	Expected
<i>Mycobacterium smegmatis</i> F <sub>420</sub> -5.3 <sup>b</sup>	5	8.74 (s)	0.94	1.0
	6	7.82 (d)	1.00	1.0
	7+9	6.89 (m)	2.02	2.0
	1'-5'+Glu methine	3.8–5.1 (m) <sup>c</sup>	10.56	12.3
	Glu $\gamma$ -methylene	2.34 (m)	10.06	10.6
	Lactyl methyl	1.49 (d)	2.78	3.0
<i>Methanobacterium thermoautotrophicum</i> F <sub>420</sub> -2	5	8.68 (s)	1.03	1.0
	6	7.79 (d)	1.00	1.0
	7+9	6.85 (m)	2.02	2.0
	1'-5'+Glu methine	3.7–5.1 (m) <sup>c</sup>	6.45	9.0
	Glu $\gamma$ -methylene	2.35 (m)	4.17	4.0
	Lactyl methyl	1.49 (d)	3.07	3.0

<sup>a</sup>The number of protons in the integration, normalized with C-6 equal to one

<sup>b</sup>The F<sub>420</sub> used for NMR was analyzed by HPLC and found to contain principally what are thought to be F<sub>420</sub> with 5- or 6-glutamate residues. The calculated average corresponds to 5.3 glutamate residues

<sup>c</sup>This region includes seven non-OH protons from the ribityl molecule and one methine proton from each glutamate residue; the water proton peak at 4.85 ppm was subtracted, leading to more potential error than with other assignments

### HPLC analysis of $F_{420}$ from mycobacteria and methanogens

Figure 3 provides examples of HPLC profiles of samples extracted from three microorganisms. C-18 reverse-phase HPLC has been used previously to distinguish  $F_{420}$  species hypothesized to have varying numbers of glutamate residues; the more glutamate residues, the more charge

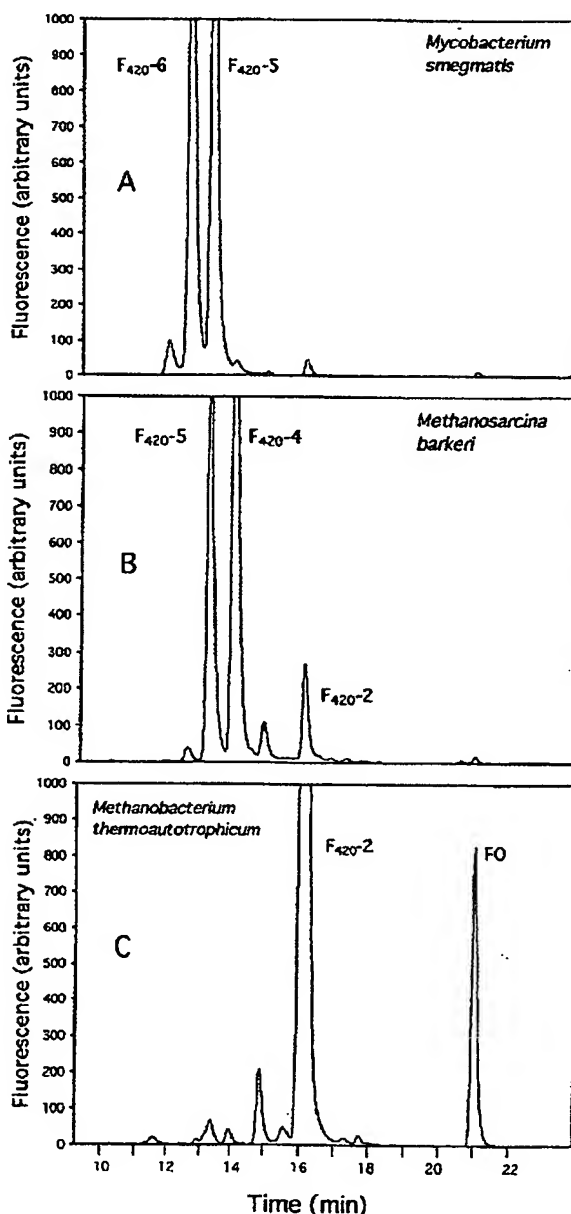


Fig. 3 HPLC elution profiles of extract prepared from A *Mycobacterium smegmatis*, B *Methanosarcina barkeri*, and C *Methanobacterium thermoautotrophicum*

and the shorter the retention time (Gorris and van der Drift 1986, 1988; Peck 1989). The deazaflavin with the least charge, FO, binds most tightly. *Methanobacterium thermoautotrophicum* extract contained principally  $F_{420-2}$ , consistent with structural NMR data above, and published information (Eirich et al. 1978), as well as a moderate amount of FO and a small amount of what we believe is  $F_{420-3}$ . *Methanosarcina barkeri* extract contained mostly a mixture of  $F_{420-4}$  and  $F_{420-5}$ , in agreement with its anticipated composition (Gorris and van der Drift 1986; Peck 1989). *M. smegmatis* extract contained mostly a mixture of  $F_{420-5}$  and  $F_{420-6}$ , in agreement with our NMR data above, as well as a small amount of what we believe to be  $F_{420-7}$ .

Table 2 provides quantitative HPLC data on these organisms and a range of other mycobacteria. Of particular interest, all *Mycobacterium* species, including *M. avium*, have major amounts of only  $F_{420-4}$  to  $F_{420-7}$ ; five and six glutamate residue forms generally predominate. In the mycobacteria, FO accounted for about 1-7% of the total deazaflavin. There is no evidence for significant levels of  $F_{420-1}$  in any *Mycobacterium* species. This is in contrast to a previous report that  $F_{420-1}$  was the sole  $F_{420}$  species in *M. avium* (Naraoka et al. 1984). Due to this conflict, we examined *M. smegmatis* grown in the medium with high glutamate levels used by Naraoka et al. (1984) to see if  $F_{420-1}$  was predominant or detectable. As shown in Table 2, this was not the case. If glutamate-free medium was used, the major structures were still  $F_{420-5}$  and  $F_{420-6}$  (data not shown). We believe that the incorrect assignment of the  $F_{420}$  structure in *M. avium* by Naraoka et al. et al. (1984) was due to acid hydrolysis that removed most glutamate groups, as a result of the use of HCl as a chromatographic eluant, particularly if a fraction was concentrated by rotary evaporation.

### Degradation of $F_{420}$ by peptidases

Several peptidases have been used by others to evaluate glutamate linkages in folic acid and  $F_{420}$ . Carboxypeptidase Y is reported to cleave only  $\alpha$ -glutamyl bonds (bonds between the NH-group of a glutamate and the  $\alpha$ -carboxyl of the previous glutamate), and has been used with *E. coli* folic acid (Ferone et al. 1986; Johnson et al. 1988). Incubation of  $F_{420}$  with carboxypeptidase Y from two different suppliers failed to show  $F_{420}$  degradation. This suggests that no  $\alpha$ -glutamyl bonds are present, but it is possible that  $F_{420}$ , as opposed to folic acid, is a poor substrate.

Commercially available crude preparations of  $\gamma$ -glutamyltranspeptidase have been used to cleave glutamyl-glutamyl bonds in  $F_{420}$  (Lin and White 1986) and in folates (Blakley and Benkovic 1984). We also found that glutamate residues were slowly cleaved from *M. smegmatis*  $F_{420}$  by these preparations, to yield (after 24-48 h) three small  $F_{420}$  derivatives that are not FO, but which may be  $F_{420-1}$ , FO-phosphate, and  $F_{420-0}$  (FO-phosphate-lactyl), based on their elution between  $F_{420-2}$  and FO (data not shown). Unfortunately, purified enzymes of this

**Table 2** Relative amounts of  $F_{420}$  types and FO in several *Mycobacterium* species and in two methanogen species. The total HPLC peak area of the  $F_{420}$  species, plus FO, add up to 100%. The numbers 7 to 2 refer to the numbers of glutamate molecules on  $F_{420}$ , corresponding to  $F_{420-7}$  to  $F_{420-2}$ . Very small peaks that may represent  $F_{420-1}$  and  $F_{420-8}$  are not included

Organism	Relative amounts of $F_{420}$ species and FO in cells (percent of total $F_{420}+FO$ )						
	7	6	5	4	3	2	FO
<i>Methanosarcina barkeri</i>	0.1	1.5	39.4	49.2	3.5	5.7	0.6
<i>Methanohacterium thermoautotrophicum</i>	0.38	0.12	0.55	0.75	3.7	80.3	14.4
<i>Mycobacterium smegmatis</i>	3.9	45.8	46.8	2.0	0.4	0.8	0.4
<i>Mycobacterium smegmatis</i> (high Glu)	3.5	33.6	51.5	7.4	2.2	0.2	1.7
<i>Mycobacterium avium</i>	28.0	40.4	24.0	0.0	0.4	0.3	6.9
<i>Mycobacterium bovis</i> BCG	9.6	36.2	35.4	10.8	2.6	2.8	2.6
<i>Mycobacterium tuberculosis</i> H37Rv	3.1	36.5	42.8	8.7	3.8	1.9	3.3
<i>Mycobacterium tuberculosis</i> CSU93	2.9	34.7	39.1	11.1	5.8	2.3	4.2
<i>Mycobacterium fortuitum</i>	0.4	14.4	62.8	15.4	3.7	1.5	1.8

type are not available from commercial suppliers, and since these peptidases were crude preparations, other activities could have been present, reducing the certainty that only  $\gamma$ -linked glutamate groups were present. As an alternative, we obtained a partly purified  $\gamma$ -glutamyl hydrolase (carboxypeptidase G) from *Pseudomonas* species that is reported to remove glutamate from methotrexate and folic acid, and to cleave peptides containing L-glutamate and not D-glutamate (Goldman and Levy 1967; Levy and Goldman 1967). This enzyme removed glutamate groups from  $F_{420-5}$  and  $F_{420-6}$  to form only one HPLC product consistent with an  $F_{420}$  derivative with one or no glutamate molecule (based on its retention time between  $F_{420-2}$  and FO). We purified 16 nmol of this product, and determined by amino acid analysis that it contained no glutamate residue. Although its precise structure is unknown, this product is in agreement with the removal of the sole glutamate molecule from methotrexate and folic acid by carboxypeptidase G to yield a free carboxyl group and free glutamate, as observed previously (Goldman and Levy 1967; Levy and Goldman 1967). We speculate that  $F_{420}$  is converted by carboxypeptidase G to  $F_{420-0}$ , which contains the phosphate and lactyl groups, but no glutamate. Thus, our data showing that glutamate residues on  $F_{420}$  are not removed by carboxypeptidase Y, but are fully removed by carboxypeptidase G, are consistent with a  $\gamma$ -linked L-glutamate chain on *Mycobacterium*  $F_{420}$ , as depicted in Fig. 1B. Also of importance to our work is that the small  $F_{420}$  derivatives produced by  $\gamma$ -glutamyltranspeptidase degradation, and the putative  $F_{420-0}$  produced by carboxypeptidase G, may be valuable molecules for the assay of enzymes of the  $F_{420}$  biosynthesis pathway that add glutamate or lactyl groups.

**Acknowledgements** This work was supported by NIH grant GM 55552 and USDA grant 4132008 to L.D. We appreciate the preparation of *M. tuberculosis* by Dr. John Belisle, assistance from Dr. William Kearney of the NMR Facility, and help from Dr. Elena Rus of the Molecular Analysis Facility.

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